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(21) International Application Number: PCT/US90/06178 (22) International Filing Date: 26 October 1990 (26.10.90) (30) Priority data: 427,321 26 October 1989 (26.10.89) US (71) Applicant: SRI INTERNATIONAL [US/US]; 333 Ravenswood Avenue, Menlo Park, CA 94025-3493 (US). (71)(72) Applicant and Inventor: TSIEN, Roger, Y. [US/US]; 8535 Nottingham Place, La Jolla, CA 92037-2125 (US). (72) Inventors: ROSS, Pepi ; 745 Contra Costa Avenue, Berkeley, CA 94707 (US). FAHNESTOCK, Margaret ; 2724 Gamble Court, Hayward, CA 94542 (US). JOHNSTON, Allan, J. ; 909 North California Avenue, Palo Alto, CA 94303 (US).		(74) Agents: CHEN, John, Y.; SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025-3493 (US) et al. (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DNA SEQUENCING (57) Abstract <p>The present invention relates to an instrument and a method to determine the nucleotide sequence in a DNA molecule without the use of a gel electrophoresis step. The method employs an unknown primed single stranded DNA sequence which is immobilized or entrapped within a chamber with a polymerase so that the sequentially formed cDNA can be monitored at each addition of a blocked nucleotide by measurement of the presence of an innocuous marker on specified deoxyribonucleotides. The invention also relates to a method of determining the unknown DNA nucleotide sequence using blocked deoxynucleotides. The blocked dNTP has an innocuous marker so that its identity can be easily determined. The present instrument and method provide a rapid accurate determination of a DNA nucleotide sequence without the use of gel electrophoresis.</p>		

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DNA SEQUENCING

Background Of The Invention

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Field of the Invention

This invention relates to DNA sequencing. More particularly, it relates to methods and apparatus for determining the sequence of deoxyribonucleotides within DNA molecules.

Description of Background Art

DNA sequencing is an important tool. A current goal of the biological community in general is the determination of the complete structure of the DNA of a number of organisms, including man. This information will aid in the understanding, diagnosis, prevention and treatment of disease.

Current DNA sequencing methods employ either chemical or enzymatic procedures to produce labeled fragments of DNA molecules. In the chemical method, reactions are performed that specifically modify certain of the nucleotide bases present in the end-labeled DNA. These reactions are carried out only partially to completion so that only a portion of the bases present in the molecules are reacted. These modified bases are then treated with piperidine, to cleave the DNA chains at the modified bases producing four sets of nested fragments. These fragments are then separated from one another according to size by electrophoresis in polyacrylamide gels. The fragments can then be visualized in the gels by

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means of radioactive labels. The position of the fragments in the gel indicates the identity of the last nucleotide in each fragment so that on the gel a "ladder" of fragments, with each step identified, is assembled to
5 provide the overall sequence.

In the enzymatic method, the DNA to be sequenced is enzymatically copied by the Klenow fragment of DNA polymerase I or by a similar polymerase enzyme such as Taq polymerase or Sequenase™. The enzymatic copying is carried
10 out in quadruplicate. In each of the four reactions a low concentration of a chain terminating dideoxynucleotide is present, a different dideoxynucleotide being present in each of the four reactions (ddATP, ddCTP, ddGTP and
15 ddTTP). Whenever a dideoxynucleotide is incorporated, the polymerase reaction is terminated, again producing sets of nested fragments. Again, the nested fragments have to be separated from one another by electrophoresis to determine the sequence.

Recently, new advances in sequencing technology
20 have introduced automated methods. Applied Biosystems has developed an instrument based on the use of fluorescent labels and a laser-and computer-based detection system (Smith et al., 1986; Smith, 1987). An automated system developed by E.E. du Pont de Nemours & Company, Inc.
25 (Prober et al., 1987) is similar to the Applied Biosystems instrument but uses fluorescently labeled ddNTPs to terminate the reaction instead of fluorescent primers. Hitachi (Japan) and EMBL (West Germany) have developed similar systems (Ansorge et al., 1986). Other approaches
30 involve multiplexing technology (Church and Kieffer-Higgins, 1988), detection of radioactively labeled DNA fragments by sensitive Beta-detectors (EG&G), automated gel readers (BioRad), and automated liquid
handlers (Beckman Instruments; Seiko; Goodenow, University
35 of California, Berkeley).

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The need to rely on electrophoresis and a separation according to size as part of the analytical scheme is a severe limitation. The gel electrophoresis is a time-consuming step and requires very highly trained skilled personnel to carry it out correctly. The present invention provides methods and apparatus for sequencing DNA which do not require electrophoresis or similar separation according to size as part of their methodology.

10 References of Interest

The following articles and patents relate to the general field of DNA sequencing and are provided as a general summary of the background art. From time to time reference will be made to these items for their teaching of synthetic methods, coupling and detection methodologies, and the like. In these cases, they will generally be referred to by author and year.

- W.B. Ansorge, et al., (1987) Nucleic Acid Research, 15:4593-4602.
- 20 W.B. Ansorge, et al., (1986) Journal of Biochemical and Biophysical Methods, 13:325-323.
- J. T. Arndt-Jovin, et al., (1975) European Journal of Biochemistry, 54:411-413.
- H. Bunemann, et al. (1982) Nucleic Acids Research, 10:7163-7180.,
- 25 L.D. Cama, et al., (1978) Journal of the American Chemical Society, 100:8006.
- G. M. Church, et al., (1988) Science 240:185-188.
- 30 S.A. Chuvpilo, et al., (1984) "A Simple and Rapid Method for Sequencing DNA," FEBS 179:34-36.
- L.F. Clerici, et al., (1979) Nucleic Acids Research, 6:247-258.
- L.A. Cohen, et al., (1966) Journal of Organic Chemistry, 31:2333.
- 35

-4-

- B.A. Connolly, (1987) Nucleic Acids Research, 15:3131-3139.
- C.G. Cruse, et al., (1978) Journal of Organic Chemistry, 43:3548-3553.
- 5 P.T. Englund, et al., (1969) Journal of Biological Chemistry, 244:3038-3044.
- B.C. Froehler, et al., (1986) Nucleic Acids Research, 14:5399-5407.
- R. Gigg, et al., (1968) Journal of the Chemical
10 Society, C14:1903-1911.
- P.T. Gilham, (1968) Biochemistry, 7:2809-2813.
- M.L. Goldberg, et al., (1979) Methods in Enzymology, 68:206-220.
- T. Goldkorn, et al., (1986) Nucleic Acids
15 Research 14:9171-9191.
- T.W. Greene, (1981) Protective Groups in Organic Synthesis, John Wiley and Sons, Inc., New York, New York.
- E. Hansbury, et al., (1970) Biochemical & Biophysical Acta, 199:322-329.
- 20 C. Hansen, et al., (1987) Analytical Biochemistry, 162:130-136.
- W.D. Henner, et al., (1983) Journal of Biological Chemistry, 258:151198-15205.
- J.A. Huberman, et al., (1970) Journal of
25 Biological Chemistry, 245:5326-5334.
- Y. Kanaoka, (1977), Angewante Chemie International Edition English, 16:137-147.
- A. Kornberg, (1974), DNA Synthesis, W. H. Freeman and Company, San Francisco.
- 30 A.A. Kraevskii, et al., (1987) Molecular Biology, 21:25-29.
- A.A. Kraevsky, et al., (1987) Biophosphates and Their Analogues--Synthesis, Structure, Metabolism and Activity, K.S. Bruzik and W.J. Stec (Eds.), Elsevier,
35 Amsterdam, pp. 379-390 (and references therein).

-5-

- J.N. Kremsky, et al., (1987) Nucleic Acids Research, 15:2891-2909.
- T.V. Kutateladze, et al., (1987) Molecular Biology, 20:222-231.
- 5 J.A. Langdale, et al., (1985) Gene 36:201-210.
- R.T. Letsinger, et al. (1964) Journal of Organic Chemistry, 29:2615-2618.
- J.K. Mackey, et al., (1971) Nature, 233:551-553.
- T. Maniatis, et al., (1982) Molecular Cloning, A
- 10 Laboratory Handbook, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- A. M. Maxam, et al., (1980) Methods in Enzymology, 65:499-560.
- E. Ohtsuka, et al., (1978) Journal of the
- 15 American Chemical Society, 100:8210-8213.
- A.V. Papchikhin, et al., (1985) Bioorganic Chemistry, 11:716-727.
- S. Pochet, et al., (1987), Tetrahedron, 43:3481-3490.
- 20 R. Polsky-Cynkin, et al., (1985) Clinical Chemistry, 31:1438-1443.
- J.M. Prober, et al., (1987) Science, 238:336-341.
- C.B. Reese, et al. (1968) Tetrahedron Letters,
- 25 40:4273-4276.
- T.A. Rezovskaya, et al., (1977) Molecular Biology, 11:455-466.
- F. Sanger, et al., (1977) Proceedings of the National Academy of Science USA, 74:5463-5467.
- 30 S.R. Sarfati, et al. (1987) Tetrahedron Letters, 43:3491-3497.
- B. Seed, (1982) Nucleic Acids Research, 10:1799-1810.
- A.J.H. Smith, (1980) Methods in Enzymology,
- 35 65:560-580.

-6-

- L.M. Smith, et al., (1986) Nature, 321:674-679.
L.M. Smith, (1987) Science, 235:G89.
E.M. Southern, (1975) Journal of Molecular Biology, 98:503-517.
- 5 S. Tabor, et al., (1987) Proceedings of the National Academy of Sciences USA, 84:4767-4771.
R.I. Zhdanov, et al., (1975) Synthesis, 1975:222-245.
- 10 Additional references of interest are:
N. Dattagupta, U.S. Patent No. 4,670,380 issued June 2, 1987.
W.J. Martin, European Patent Application No. 0187699, published July 16, 1986.
- 15 Japan Kokai Tokyo Kobo JP 58/87,452 (May 25, 1983); Chem. Abs, Vol. 99, No. 172376n.
R. Lewis, "Computerizing Gene Analyses" High Technology, December 1986, p. 46 ff.
C. Connell, et al. "Automated DNA Sequence
- 20 Analysis", BioTechniques, Vol. 5, No. 4, p. 342 ff. (1987).
J.F.M. De Rooiz, et al., Journal of Chromotography, Vol. 177, p. 380-384 (1987).

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Statement of the Invention

The present invention provides methods and apparatus for determining the sequence of

30 deoxyribonucleotides in a DNA molecule. A key characteristic of this invention is that it determines the DNA sequence without recourse to electrophoresis or other size-based separation techniques.

In one aspect, the present invention provides a

35 method for determining the deoxyribonucleotide sequence of a single stranded DNA subject molecule. This method

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involves synthesizing, in the presence of a multitude of identical copies of the subject DNA, the DNA molecule which is complementary to it. This synthesis is carried out using deoxyribonucleotide triphosphates (dNTP) in a stepwise serial manner so as to simultaneously build up numerous copies of the complementary molecule, dNTP by dNTP. As each dNTP is added to the growing complementary molecules, it is identified by way of an appropriate label (i.e., reporter group). By noting the identity of the bases present in this complementary molecule and using standard rules of DNA complementation, one can translate from the complementary molecule to the corresponding original subject molecule and thus obtain the deoxyribonucleotide sequence of the subject molecule.

15 In an additional aspect, this invention provides apparatus for carrying out the above-described method.

As will be seen in the Detailed Description of the Invention which follows, this method and apparatus for carrying it out can take many different configurations. A key to all of them, however, is the fact that the DNA sequence is determined not by generating a series of nested fragments which must be separated according to size but rather by direct identification of the dNTPs as they are incorporated into the growing complementary DNA chain.

25 This invention can be carried out in a single reaction zone with multiple differentiable reporters or in multiple reaction zones with a single reporter in each zone. It can be carried out by detecting the incremental signal change after addition of reporters or by noting each added reporter separately. The various reporters can be measured in the reaction zones while attached to the growing molecule or they can be separated from the molecule and then measured.

35 The invention can be practiced to create the growing complementary DNA chain without interruption or it can be practiced in stages wherein a portion of the

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complementary chain is created and its sequence determined; this portion of the chain is then removed; a sequence corresponding to a region of the removed chain is separately synthesized and used to prime the template
5 chain for subsequent chain growth. The latter method can be repeated as needed to grow out in portions the complete complementary chain.

10 Detailed Description of the invention

Brief Description of the Drawings

The invention will be further described with reference being made to the accompanying drawings in
15 which:

Figures 1A and 1B are schematic diagrams of the process of this invention on a molecular level.

Figure 2 is a schematic representation of one form of apparatus for practising the invention. In this
20 embodiment the DNA growth takes place in a single reaction zone. This embodiment uses separate, distinguishable reporters associated with each of the four nucleotides incorporated into the growing molecule. The four different reporters are measured after each addition to
25 detect which base has just been added to that position of the complementary chain.

Figure 3 is a schematic representation of another form of apparatus for practising the invention. This embodiment employs four reaction zones in which the
30 molecular growth is carried out in quadruplicate. In each of the four zones, a different one of the four nucleotides is associated with a reporter (with the remaining three being unlabeled) so that the identity of the nucleotide incorporated at each stage can be determined.

35 Figure 4 is a schematic representation of an adoption of the apparatus for practising the invention

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particularly adapted for carrying out the invention to grow a series of portions of the complementary molecule as opposed to a single continuous complementary molecule.

Figures 5 through 8 are pictorial representations of chemical reaction sequences which can be used to synthesize representative labeled nucleotide building blocks for use in the practice of this invention.

Organization of this Section

This Detailed Description of the Invention is organized as follows:

First, several terms are defined in a Nomenclature section.

Second, a series of Representative Apparatus Configurations and Process Embodiments for carrying out the invention are described.

Third, Materials and Reagents and Methods of Use employed in the process of the invention are set forth, including;

Enzymes and Coupling Conditions,
Blocking Groups and Methods for Incorporation,
Deblocking Methods,
Reporter Groups, their Incorporation and
Detection, and
Immobilization of Subject DNA.

Thereafter, a series of nonlimiting EXAMPLES is provided.

Nomenclature

A number of related and generally conventional abbreviations and defined terms appear in this specification and claims. The four nucleotides are at times referred to in shorthand by way of their nucleoside bases, adenosine, cytidine, guanosine and thymidine, or "A", "C", "G" and "T". Deoxynucleotide triphosphates "dNTPs" of these materials are abbreviated as dATP, dCTP,

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dGTP and dTTP. When these materials are blocked in their 3'-OH position they are shown as 3'blockeddATP, 3'blockeddCTP, 3'blockeddGTP and 3'blockeddTTP. Similarly, when they are each tagged or labeled with a common reporter group, such as a single fluorescent group, they are represented as dA'TP, dC'TP, dG'TP and dT'TP. When they are each tagged or labeled with different reporter groups, such as different fluorescent groups, they are represented as dA''TP, dC'''TP, dG''''TP and dT''''TP. As will be explained in more detail below, the fact that the indication of labeling appears associated with the "nucleoside base part" of these abbreviations does not imply that this is the sole place where labeling can occur. Labeling could occur as well in other parts of the molecule.

Representative Apparatus Configurations and Process Embodiments

In the specification and claims, reference is made to a "subject" DNA or "template" DNA to define the DNA for which the sequence is desired. In practice, this material is contained within a vector of known sequence. A primer, which is complementary to the known sequence of the vector is used to start the growth of the unknown complementary chain. Two embodiments of this process are illustrated on a molecular level in Figures 1A and 1B.

In Figure 1A, a solid support 1 is illustrated with a reactive group A attached to its surface via tether 2. This attachment can be covalent, ionic or the like. A second reactive group X, capable of bonding to group A, again via a covalent, ionic or the like bond, is attached to the 5' end of a DNA primer 4. This primer has a known DNA sequence. When coupled to the substrate via the A-X bond it forms immobilized primer 5. Primer 5 is then hybridized to template DNA strand 6 which is made up of an unknown region 7 inserted between regions 8 and 8'.

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Regions 8 and 8' are located at the 5' and 3' ends of the unknown region and have known sequences. The 8' region's known sequence is complementary to the sequence of primer 4 so that those regions hybridize to form immobilized
5 template DNA 9. Therefore the individual dNTPs are serially added to form the DNA sequence complementary to the unknown region of the template. 11 and 12 represent the first two such dNTPs incorporated into the growing molecule. These in turn provide the identity of their
10 complements 11' and 12' respectively. This growth continues until the entire complementary DNA molecule has been constructed. Completion can be noted by identifying the sequence corresponding to the 8 region of template 6.

Turning to Figure 1B, a variation of this
15 chemistry is shown in that the template 6* carries the reactive group X which bonds to the substrate via the A-X bond to form an immobilized template 5*. This is then hybridized with primer 3* to give the immobilized, primed template 9* upon which the desired adding of dNTPs takes
20 place to add units 11 and 12 and thus identify the sequence and identity of units 11' and 12'. While in the chemistry illustrated in Figure 1B reference is made to coupling template DNA 6* via an X group on its 3' end to the A group on the substrate, it will be appreciated that
25 the template DNA 6* could just as well be coupled through its 5' end. The chemistry for such an attachment is known in the art.

Referring now to Figure 2, a device 13 for carrying out the invention is shown schematically. In
30 this schematic representation, and the representation provided in Figure 2, many components such as mixers, valves and the like are omitted to facilitate a clear focus on the invention. Device 13 includes a reaction zone 14 which carries inside it a surface 15. A plurality
35 of copies of a subject primed single stranded DNA are immobilized on this surface 15. This is the strand of DNA

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for which the sequence is desired. The immobilized DNA is depicted fancifully on surface 15 as if it were present as a series of separately visible attached strands. As will be appreciated, this is not in fact the case and is only
5 done to guide the reader as to the location of the DNA strands. The reaction zone 14 may be configured to permit direct reading of reporter signals emanating from within. Examples of this configuration include equipping the reaction zone to permit measuring fluorescence or
10 luminescence through one or more transparent walls or detecting radionuclide decay. Reaction zone 14 is fitted with inlet 16 for the addition of polymerase or another suitable enzyme capable of moderating the template-directing coupling of nucleotides to one another. The
15 reaction zone is also accessed by inlet lines, 18a-18d for four differently labeled blocked dNTPs, that is 3'blockeddA'TP, 3'blockeddC''TP, 3'blockeddG'''TP, and 3'blockeddT''''TP. These materials can be added in four separate lines, as shown, or can be premixed, if desired,
20 and added via a single line. Buffer and other suitable reaction medium components are added via line 20.

In practice, the polymerase and the four labeled dNTPs are added to the reaction zone 14 under conditions adequate to permit the enzyme to bring about addition of
25 the one, and only the one, of the four labeled blocked dNTPs which is complementary to the first available template nucleotide following the primer. The blocking group present on the 3'-hydroxyl position of the added dNTP prevents inadvertent multiple additions. After this
30 first addition reaction is complete, the liquid in reaction zone 14 is drained through line 22 either to waste, or if desired to storage for reuse. The reaction zone and the surface 15 are rinsed as appropriate to remove unreacted, uncoupled labeled blocked dNTPs. At
35 this point the first member of the complementary chain is now in place associated with the subject chain attached to

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surface 15. The identity of this first nucleotide can be determined by detecting and identifying the label attached to it.

This detection and identification can be carried out in the case of a fluorescent label by irradiating the surface with a fluorescence-exciting beam from light source 24 and detecting the resulting fluorescence with detector 26. The detected fluorescence is then correlated to the fluorescence properties of the four different labels present on the four different deoxynucleotide triphosphates to identify exactly which one of the four materials was incorporated at the first position of the complementary chain. This identity is then noted.

In the next step, a reaction is carried out to remove the blocking group and label from the 3' position on the first deoxynucleotide triphosphate. This reaction is carried out in reaction zone 14. A deblocking solution is added via line 28 to remove the 3' hydroxyl labeled blocking group. This then generates an active 3' hydroxyl position on the first nucleotide present in the complementary chain and makes it available for coupling to the 5' position of the second nucleotide. After completion of the deblocking, removal of the deblocking solution via line 22 and rinsing as needed, the four blocked, labeled deoxynucleotide triphosphates, buffer and polymerase are again added and the appropriate second member is then coupled into the growing complementary chain. Following rinsing, the second member of the chain can be identified based on its label.

This process is then repeated as needed until the complementary chain has been completed. At the completion of the construction of the complementary chain, the sequence of incorporated deoxynucleotides is known, and therefore so is the sequence of the complement which is the subject chain.

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It will be appreciated that this process is easily automated. It is a series of fluid additions and removals from a reaction zone. This can be easily accomplished by a series of timer-controlled valves and the like. This technology has been well developed in the area of oligonucleotide synthesizers, peptide synthesizers, and the like. In such an automated system, the timing can be controlled by a microprocessor or, in most cases, by a simple programmable timer. The rate and extent of reaction can be monitored by measurement of the reporter concentration at various stages.

The labels present in the blocked dNTPs can be incorporated in one of several manners. For one, they can be incorporated directly and irremovably in the deoxynucleotide triphosphate unit itself. Thus, as the complementary chain grows there is a summing of signals and one identifies each added nucleotide by noting the change in signal observed after each nucleotide is added.

Alternatively, and in many cases preferably, the label is incorporated within the blocking group or is otherwise incorporated in a way which allows it to be removed between each addition. This permits the detection to be substantially simpler in that one is noting the presence of one of the four reporter groups after each addition rather than a change in the sum of a group of reporter groups.

In the embodiment shown in Figure 2, the presence of reporter signal is noted directly in the reaction zone 14 by the analytical system noted as source 24 and detector 26. It will be appreciated, however, that in embodiments where the reporter group is removed during each cycle, it is possible to read or detect the reporter at a remote site after it has been carried out of the reaction zone 14. For example, drain line 22 could be valved to a sample collector (not shown) which would isolate and store the individual delabeling product

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solutions for subsequent reading. Alternatively, if the nature of the label permitted, the various removed labels could be read as they flowed out of the reaction zone by equipping line 22 with an in-line measurement cell such as source 24' and detector 26' or the like.

A second embodiment of this invention employs four separate parallel reaction zones. This method has the advantage of requiring only one type of labeling and being able to use it with all four dNTPs. Figure 3 shows a schematic representation of a device 30 which has the four reaction zone configuration. In this configuration there are four reaction zones 32a through 32d, each of which resembles the reaction zone 14 in Figure 2. In these cases each of the four reaction zones contains a surface 34a-d to which is immobilized numerous copies of a primed subject single stranded DNA. Each reaction zone is supplied with polymerase via lines 36a-d. Each zone is supplied with suitable reaction medium via lines 38a-38d. The four dNTPs are supplied in blocked form to each zone, as well. In zone 32a one of the blocked dNTPs is labeled, for example "A'"; in zone 32b a second dNTP is labeled, for example "C'"; in zone 32c a third dNTP is labeled, for example "G'"; and in 32d the fourth labeled dNTP "T'" is present. These labeled materials are supplied via lines 40a through 40d respectively. Unlabeled blocked dNTPs are supplied via lines 42a-d so that each of the four reaction zones contains three unlabeled blocked dNTPs and one labeled blocked dNTP. Again, as noted with reference to Figure 2, the various labeled and unlabeled dNTP's can be premixed. These premixed materials can be added to the various reaction zones via single addition lines.

Using the same general methodology described with reference to Figure 2, the single stranded DNA hybridized to a primer and attached to each of surfaces 34a-34d is contacted with polymerase (supplied via lines 36a-36d), buffer (supplied via lines 38a-38d) and the four

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bases in each of the four reaction zones. The blocked dNTP which complements the first base on the subject chain couples. In one of the four reaction zones, this base is labeled. By noting in which of the four zones this label
5 is incorporated into the growing chain, one can determine the identity of the dNTP which is incorporated at the first position. This determination of the identity of the first unit of the chain can be carried out using signal sources and detectors such as 44a-44d and 46a-46d,
10 respectively. Deblocking is carried out by adding deblocking solution to the reaction zone through lines 48a-48d. Lines 50a-50d are drain lines for removing material from the reaction zones following each step.

In this second configuration, all of the
15 variations noted with reference to the device described in Figure 2 can also be used including cumulating reporter signals and generating reporter signals away from the reaction zone by removing the reporter groups as part of each of the sequential couplings. Clearly, this
20 embodiment can be readily automated, as well.

One obvious potential shortcoming of the present invention is that it employs a long sequence of serial reactions. Even if the efficiency and yield of each of these reactions are relatively high, the overall yield
25 becomes the product of a large number of numbers, each of which is somewhat less than 1.00, and thus can become unacceptably low. For example if the yield of a given addition step is 98% and the deblocking is 98% as well, the overall yield after 15 additions is 48%, after 30
30 additions it is 23% and after 60 additions it is 5.3%.

This limitation can be alleviated by periodically halting the DNA molecule growth and using the sequence data obtained prior to halting the growth to externally recreate a portion of the molecule which can
35 then be used as a primer for renewed DNA fabrication.

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This process is illustrated in Figure 4. Figure 4 shows a schematic of an automated sequencer 52 employing the present invention. Sequencer 52 has a single reaction zone 14 combining the subject primed DNA, immobilized therein such as on surface 15. The four 3-blocked dNTP's, suitably detachably labeled, are fed to the reaction zone through line 18. Polymerase and buffers are added via lines 16 and 20, respectively. Additionally, the dNTP's, polymerase and buffer can be recycled from step to step via lines 54 and 56 and holding vessel 58. All of the valves admitting and removing fluids from reaction zone 14 can be controlled by central computer 60 which functions as a valve control clock. This computer 60 can also control the addition of deblocker from line 28, deblocking eluent with cleaved labels (as obtained when the label is present in the blocking group) is removed via line 22 and detected via detector system 24/26 reading label values in detector vessel 62.

This embodiment illustrates the use of a fluorescent label system and shows the addition of fluorescent sensitizer (flooder) via line 64 to the fluorescent detection zone 62.

Following detection of the label in vessel 62, the deblocking solution and detected label are discarded via line 66.

The signal presented by the label identified by detector 26 is passed to analog/digital converter 68 and therein to a memory in central computer 60 where it is stored. After a number of iterations, the memory in computer 60 contains the sequence of an initial portion of the complementary DNA molecule which has been constructed in association with the subject or target DNA molecule contained within reactor 14. After some number of units have been assembled - typically 25 to 300, or more; preferably 50 to 300, or more; and more preferably 100 to 300, or more - the growing complementary DNA molecule is

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stripped from the immobilized subject DNA molecule and discarded. This stripping (denaturing) can be done by art-known methods such as by warming the reaction zone to 75°C or higher (preferably 90-95°C) for a few (1-15) minutes. Other equivalent methods can be used. The sequence information stored in computer 60 is used to drive DNA synthesizer 70 to externally create a new DNA primer corresponding to at least a portion of the discarded DNA molecule. (The sequence can also be read on printer 72, if desired.) This newly constructed DNA primer molecule is fed through line 74 to reaction zone 14 under hybridization conditions so as to join to the complementary region of the subject DNA molecule as a new primer.

The length of the primer must be adequate to unambiguously and strongly hybridize with a single region of the subject DNA. As is known in the hybridization art, this can depend upon factors such as the sequence, environmental conditions, and the length of the subject DNA. For efficiency of operation, the primer should ideally be as short as possible. Primer lengths typically range from about 10 bases to about 30 bases, although shorter primers would certainly be attractive if they met the above criteria, and longer primers could be used albeit with an increase in cost and time. Good results generally are achieved with primers from 12 to 20 bases long. This gives the molecular growth reaction a "new start" with a large number of properly primed identical molecules. This allows a strong signal to be generated when the next dNTP is coupled.

This restarting of the growth can be carried out as often as needed to assure a strong consistent label signal.

Materials and Reagents and Methods of UseEnzymes and Coupling Conditions

The coupling process employed in this invention to incorporate each of the blocked deoxynucleotide triphosphates into the growing complementary chain is an enzyme moderated process. Each member of the complementary DNA chain is added using a suitable template-dependent enzyme. One enzyme which can be used is SequenaseTM enzyme (an enzyme derived from bacteriophage T₇ DNA polymerase that is modified to improve its sequencing properties - see Tabor and Richarson, Proc. Nat. Acad. Sci. USA, 84:4767-4771 (1987)--sold by United States Biochemical Corporation, Cleveland, Ohio). Other polymerases which can be used instead of SequenaseTM include but are not limited to Klenow fragment of DNA polymerase I, AMV reverse transcriptase, and Taq polymerase.

Typically the coupling conditions which are employed are those known in the art for these enzymes. In the case of SequenaseTM these include temperatures in the range of from about room temperature to about 45°C; a buffer of pH 7 to 8 and preferably pH 7.3 to 7.7; an enzyme concentration of from about 0.01 units per microliter to about 1 unit per microliter and a reaction time of from about 1 to about 20 minutes and preferable 1 to 5 minutes. A typical buffer for use with SequenaseTM is made up of

	0.040	M Tris HCl (pH 7.5)
30	0.050	M sodium chloride
	0.010	M magnesium chloride
	0.010	M dithiothreitol

In the case of Klenow fragment of DNA polymerase I, these typical conditions include temperatures in the range of from about 10°C to about 45°C and preferably from about 15°C to about 40°C; a buffer of pH 6.8 to 7.4 and

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preferably pH 7.0 to 7.4; an enzyme concentration of from about 0.01 units per microliter to about 1 unit per microliter and preferably from about 0.02 to about 0.15 units per microliter and a reaction time of from about 1 to about 40 minutes. A typical buffer for use with Klenow fragment of DNA polymerase I is made up of

0.05 M Tris chloride, pH 7.5
0.05 M magnesium chloride
0.05 M sodium chloride
0.010 M dithiothreitol

These conditions are representative. When other enzymes are employed, one should use the conditions optimal for them since it is generally desirable to run the addition reaction as quickly as possible. To this end, it is often desirable to use temperatures of 42°C for reverse transcriptase; 24°C for Klenow polymerase; 37°C with SequenaseTM and 72°C with Taq polymerase. In addition, to force the reaction, especially with derivatized dNTP's it may often be helpful to use substantial excesses (over stoichiometry) of the dNTP's, or to modify other conditions such as the salt concentration.

Blocking Groups and Methods for Incorporation

The coupling reaction generally employs 3'-hydroxyl-blocked dNTPs to prevent inadvertent extra additions.

The criteria for the successful use of 3'-blocking groups include:

- (1) the ability of a polymerase enzyme to accurately and efficiently incorporate the dNTPs carrying the 3'-blocking groups into the cDNA chain,
- (2) the availability of mild conditions for rapid and quantitative deblocking, and

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(3) the ability of a polymerase enzyme to reinitiate the cDNA synthesis subsequent to the deblocking stage.

In addition, if the 3'-blocking group carries a reporter group, it is desirable that the reporter permit sensitive detection either when part of the cDNA chain before deblocking or subsequent to deblocking in the reaction eluant.

For the present invention, 3'-blocked dNTPs are used that can be incorporated in a template-dependent fashion and easily deblocked to yield a viable 3'-OH terminus. The most common 3'-hydroxyl blocking groups are esters and ethers. Other blocking modifications to the 3'-OH position of dNTPs include the introduction of groups such as -F, -NH₂, -OCH₃, -N₃, -OPO₃⁼, -NHCOCH₃, 2-nitrobenzene carbonate, 2,4-dinitrobenzene sulfenyl and tetrahydrofuranyl ether. Incorporation and chain termination have been demonstrated with dNTPs containing many of these blocking groups (Kraevskii et al., 1987).

Presently preferred embodiments focus on the ester blocking groups such as lower (1-4 carbon) alkanolic acid and substituted lower alkanolic acid esters, for example formyl, acetyl, isopropanoyl, alpha fluoro- and alpha chloroacetyl esters and the like; ether blocking groups such as alkyl ethers; phosphate blocking groups; carbonate blocking groups such as 2-nitrobenzyl; 2,4-dinitrobenzene-sulfenyl and tetrahydrothiofuranyl ether blocking groups. Blocking groups can be modified to incorporate reporter moieties, if desired, including radiolabels (tritium, C¹⁴ or P³², for example), enzymes, fluorophores and chromophores.

These blocking materials in their fundamental forms have all been described in the literature as has their use as blockers in chemical DNA synthesis settings. Two representative blockers, esters and phosphate, can be incorporated into dNTP's as follows:

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The general procedure for synthesis of 3'-O-acyl dNTPs is outlined in Reaction Scheme 1 set forth in Figure 5 for 3'-O-acetyl TTP. 5'-Dimethoxytrityl (DMT) thymidine 2 is prepared from thymidine 1 by reaction with DMT chloride in pyridine, followed by acetylation of the 3'-OH function using acetic anhydride in pyridine to yield 3 (Zhdanov and Zhenodarova, 1975). Treatment of the 5'-DMT group with 2% benzene-sulfonic acid yields 4, which is converted into the phosphomonoester 5 by reaction with POCl₃ in trimethyl phosphate (Papchikhin et al., 1985) and by purification using chromatography. The 5'-monophosphate is converted into the 5'-triphosphate 6 by activation with N,N'-carbonyldiimidazole, followed by pyrophosphorylation with tri(n-butylammonium) pyrophosphate (Papchikhin et al., 1985) and purification by chromatography.

Preparation of 3'-O-acetyl derivatives of dATP, dCTP, and dGTP follows the same general scheme, with additional steps to protect and deprotect the primary amino functions (see below). Because 5'-triphosphate derivatives of nucleosides are often unstable, the final preparative steps outlined above may be optionally carried out just before introducing the dNTPs into the reaction cell. If radiolabeled acetic anhydride is used, this serves to introduce a label into the ester blocking group.

When carrying out this ester-blocking of the 3'-OH group it should be borne in mind that the primary amino residues in cytosine, adenine, and guanine are also susceptible to attack by electrophilic reagents such as acetic anhydride and may be advantageously protected. In chemical oligonucleotide synthesis (phosphotriester or phosphoramidite approaches), various N-acyl groups are commonly used for protection of the primary amine (Papchikhin et al., 1985). Because the N-acyls are stable in acidic and neutral solutions, removal is typically effected by ammonolysis. These conditions are likely to

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cleave 3'-O-acyl blocking groups and other blocking groups hydrolyzable under basic conditions, so alternative N-protection should be used if it is desired to selectively remove the amino group protection. Several
5 selectively-removable amine protection groups include carbamates cleavable by acid hydrolysis [t-butyl, 2-(biphenyl)isopropyl] and certain amides susceptible to acid cleavage (formamide, trichloroacetamide) (Greene, 1981).

10 The synthesis of 3'-monophosphate dNTPs is outlined in Reaction Scheme 2 set forth in Figure 6 for TTP and is a modification of reported procedures for chemical oligonucleotide synthesis using the H-phosphonate method (Froehler et al., 1986). 5'-DMT-3'-thymidine
15 H-phosphonate 7 is prepared by reaction of 5'-DMT thymidine 2 with phosphorous trichloride, 1,2,4-triazole, and N-methylmorpholine. Removal of the 5'-protecting group and formation of the 5'-triphosphate moiety (7 to 11) is achieved as shown in Scheme 1. The 3'-OH
20 phosphonate TTP 11 is converted to the 3'-O-monophosphate 12 by oxidation with iodine in basic solution.

For other nucleotide derivatives, protection of the primary amino groups is performed prior to phosphonation. In this preparation, standard amino
25 protecting groups cleavable by ammonolysis may be used.

Deblocking Methods

After successfully incorporating a 3'-blocked nucleotide into the DNA chain, the sequencing scheme
30 requires the blocking group to be removed to yield a viable 3'-OH site for continued chain synthesis. The deblocking method should:

- (a) proceed rapidly,
- (b) yield a viable 3'-OH function in high
35 yield, and,

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(c) not interfere with future enzyme function or denature the DNA strand.

(d) the exact deblocking chemistry selected will, of course, depend to a large extent upon the blocking group employed. For example, removal of ester blocking groups from the 3'hydroxyl function is usually achieved by base hydrolysis. The ease of removal varies widely; generally, the greater the electro-negativity of substituents on the carbonyl carbon, the greater the ease of removal. For example, the highly electronegative group trifluoroacetate is cleaved rapidly from 3' hydroxyls in methanol at pH 7 (Cramer et al., 1963) and thus would not be stable during coupling at that pH. Phenoxycetate groups are cleaved in less than one minute but require substantially higher pH such as is achieved with NH_3 /methanol (Reese and Steward, 1968). To prevent significant premature deblocking and DNA degradation, the ester deblocking rate is advantageously selected so as to exhibit a deblocking rate of less than 10^{-3}s^{-1} during the incorporation, and at least 10^{-1}s^{-1} during the deblocking stage. Ideally, this rate change is achieved by changing the buffer pH from 7 to about 10, but care must be taken not to denature the DNA.

A wide variety of hydroxyl blocking groups are cleaved selectively using chemical procedures other than base hydrolysis. 2,4-Dinitrobenzenesulfonyl groups are cleaved rapidly by treatment with nucleophiles such as thiophenol and thiosulfate (Letsinger et al., 1964). Allyl ethers are cleaved by treatment with Hg(II) in acetone/water (Gigg and Warren, 1968). Tetrahydrothiofuranyl ethers are removed under neutral conditions using Ag(I) or Hg(II) (Cohen and Steele, 1966; Cruse et al., 1978). These protecting groups, which are stable to the conditions used in the synthesis of dNTP analogues and in the sequence incorporation steps, have some advantages over groups cleavable by base hydrolysis -

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deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.

Photochemical deblocking can be used with
5 photochemically-cleavable blocking groups. Several blocking groups are available for such an approach. The use of o-nitrobenzylethers as protecting groups for 2'-hydroxyl functions of ribonucleosides is known and demonstrated (Ohtsuka et al., 1978); removal occurs by
10 irradiation at 260 nm. Alkyl o-nitrobenzyl carbonate protecting groups are also cleaved by irradiation at pH 7 (Cama and Christensen, 1978).

Enzymatic deblocking of 3'-OH blocking groups is also possible. It has been demonstrated that T4
15 polynucleotide kinase can convert 3'-phosphate termini to 3'-hydroxyl termini that can then serve as primers for DNA polymerase I (Henner et al., 1983). This 3'-phosphatase activity is used to remove the 3'-blocking group of those dNTP analogues that contain a phosphate as the blocking
20 group; the radioactive label enables the incorporation of the nucleotide analogue and the removal of the phosphate group to be followed easily. If the use of radioisotopes represents too great a drawback, it is possible to use unlabeled phosphate monoesters with a cleavable
25 fluorescent label (see below).

This method is improved by increasing the efficiency and speed of each step. Upon selection of the optimal methodology for incorporation and deblocking, other nonchemical assistance may be used to accelerate
30 chemical deblocking. This may include, for example applying controlled ultrasonic irradiation of the reaction chamber to increase the rate of the deblocking step if mass transport limitations are significant and raising the reaction temperature up to about 50°C for a short period.

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Reporter Groups, their Incorporation and Detection

As part of this invention, the incorporation of each dNTP into the complementary chain is noted by detecting a label or reporter group present in or
5 associated with the incorporated dNTP. The labels or markers are "innocuous". An "innocuous marker or label or reporter" refers to a radioactive, fluorescent, or the like marker or reporter which has physical and chemical properties which do not interfere with either the
10 enzymatic addition of the marked nucleotide to the cDNA, or the subsequent deblocking to yield a viable 3'-OH terminus.

One simple labeling approach is to incorporate a radioactive species within the blocking group or in some
15 other location of the dNTP units. This can be done easily by C¹⁴ labeling or P³² labeling.

Another labeling approach employs fluorescent labels. These can be attached to the dNTP's via the 3'-OH-blocking groups or attached in other positions. There are
20 two general routes available using fluorescent tags:

(1) the use of a labeling group that is itself fluorescent and detected either before or after deblocking, and

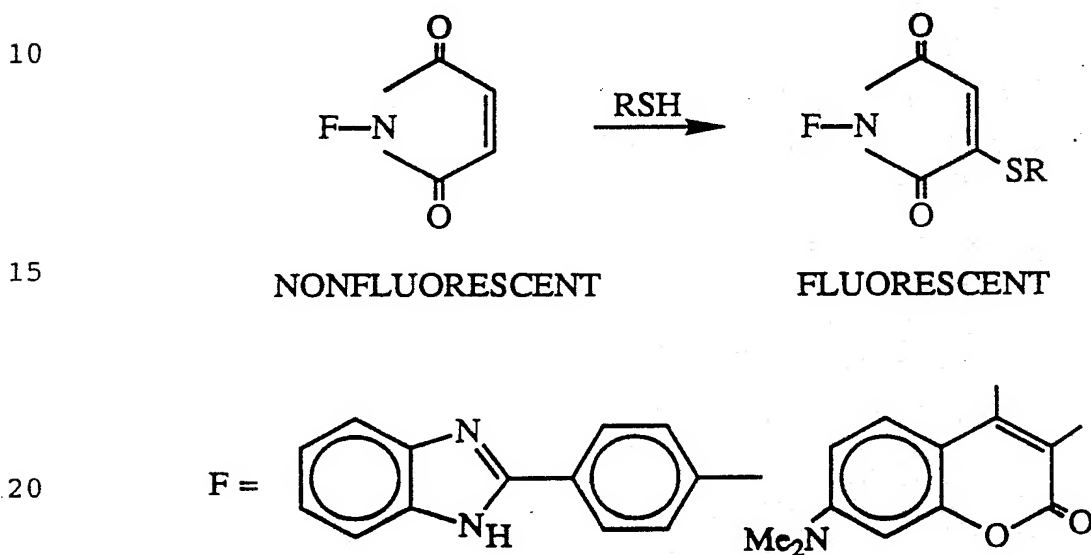
(2) the use of a nonfluorescent labeling group
25 that is detected by its fluorescent interaction with a nonfluorescent probe or other moiety.

The first route is fairly straightforward and can employ a range of known fluorophores such as rhodamines, fluoresceins and the like, typically including
30 those fluorophores known as useful in labeling dNTP's and the like. One caution however, is to try to select fluorophores which are not so large and bulky that the labeled dNTP can not be incorporated readily into the growing DNA chain by a polymerase or similarly functioning
35 enzyme. The second route can employ a fluorophore where only a fragment is attached to the dNTP. This can reduce

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size and minimize steric interference. In the second route, rapid reaction of a normally nonfluorescent probe or molecule with specific functional group(s) found only on the label fragment leads to the formation of a fluorescent addition product. This leads to a signal only when the particular labeling group is present.

One system that is applicable to this scheme is the thiol/maleimide interaction:



Certain N-substituted maleimides which are normally nonfluorescent react readily with various thiols to form fluorescent products (Kanaoka, 1977). Blocking groups or other label fragment groups containing free thiol functions, such as $\text{-COCH}_2\text{SH}$, can be used for this approach. Alternatively, the blocking group or other label fragment can contain a metal-binding ligand, e.g. a carboxylic acid group which will react with added rare earth metal ions such as europium or terbium ions to yield a fluorescent species.

While the above-described approaches to labeling focus on incorporating the label into the 3'-hydroxyl blocking group, there are a number of alternatives - particularly the formation of a 3'-blocked dNTP analogue

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containing a label such as a fluorescent group coupled to a remote position such as the base. This dNTP can be incorporated and the fluorescence measured and removed according to the methods described below.

5 One method involves the use of a fluorescent tag attached to the base moiety. The tag may be chemically cleaved (either separately from or simultaneously with the deblocking step) and measured either in the reaction zone before deblocking or in the reaction eluant after
10 cleavage. This method is included because a number of base moiety derivatized dNTP analogues have been reported to exhibit enzymatic competence. Sarfati et al, (1987) demonstrates the incorporation of biotinylated dATP in nick translations, and other biotinylated derivatives such
15 as 5-biotin (19)-dUTP (Calbiochem) are incorporated by polymerases and reverse transcriptase. Prober et al. (1987) show enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and SequenaseTM.

 In another type of remote labeling the
20 fluorescent moiety or other innocuous label can be attached to the dNTP through a spacer or tether. The tether can be cleavable if desired to release the fluorophore or other label on demand. There are several cleavable tethers that permit removing the fluorescent
25 group before the next successive nucleotide is added--for example, silyl ethers are suitable tethers which are cleavable by base or fluoride, allyl ethers are cleavable by Hg(II), or 2,4-dinitrophenylsulfenyls are cleavable by thiols or thiosulfate. Cleavages using acidic conditions
30 are undesirable because DNA is more labile in acid than in base. Long tethers may be used so that the large fluorescent groups are spaced sufficiently far away from the base and triphosphate moieties and do not interfere with the binding of the dNTP to the polymerase or with
35 proper base pairing during complementary chain growth.

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Typical tethers are from about 2 to about 20, and preferably from about 3 to about 10 atoms in length.

The C-8 position of the purine structure presents an ideal position for attachment of a label.

5 Sarfati et al. (1987) describes a derivatization of deoxyadenosine at C-8 of the purine to prepare, ultimately, an 8-substituted biotin aldyldamino dATP. The Sarfati et al. (1987) approach can be used to prepare the appropriate fluorescent, rather than biotinylated,
10 analogues. A number of approaches are possible to produce fluorescent derivatives of thymidine and deoxycytidine. One quite versatile scheme is based on an approach used by Prober et al. (1987) to prepare ddNTPs with fluorescent tags. Structures A, B, C and D below illustrate the type
15 of fluorescent dNTPs that result from these synthetic approaches. The synthetic routes have a great flexibility in that the linker can be varied with respect to length or functionality. The terminal fluorescent moiety can also be varied according to need.

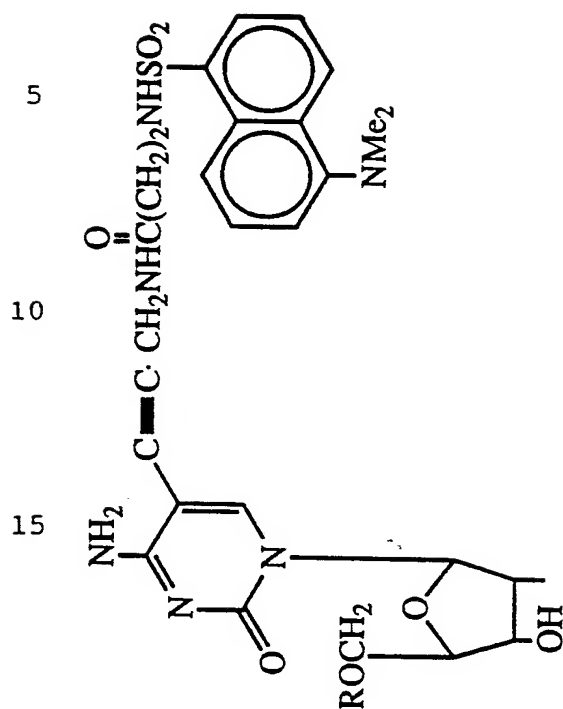
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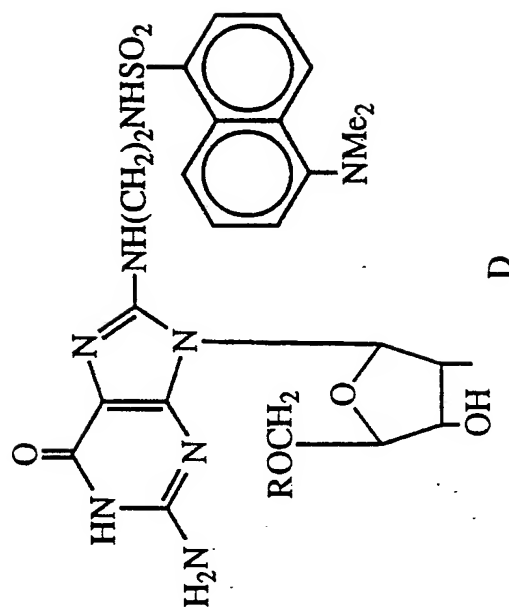
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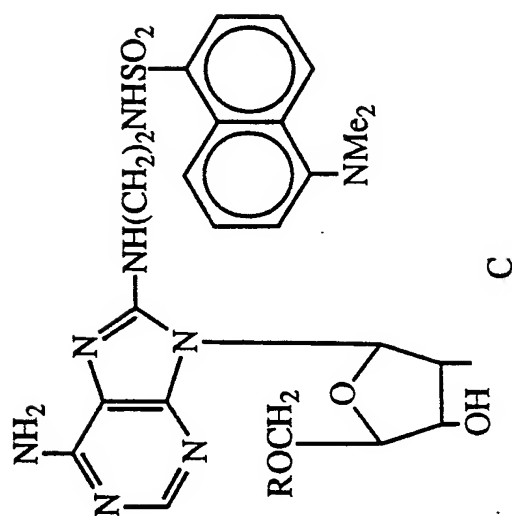


B



D

A



C

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The labels so incorporated in the growing cDNA chain are detected by conventional analytical methods. In many cases, particularly with fluorescent labels, increased detection sensitivity is a major advantage of the present method. When the fluorescent signal is detected in sequencing gels, the signal is based on a low level of fluorophores and is superimposed on a background of scatter from the gel and glass plates. This decreases sensitivity and often constrains current methods to the use of laser illumination to maximize sensitivity (Smith et al., 1986; Prober et al., 1987; Ansorge et al., 1986). Detection of fluorophores is readily achievable in commercial non-excited spectrofluorometers, such as are sold by Perkin-Elmer. In these devices, the requirement for a laser light source is eliminated (although one can of course be used if desired) allowing use of light-emitting diodes (LED) or a conventional xenon arc lamp, the choice being dictated primarily by the fluorochromes decided upon and the excitation frequency they require.

Typical LEDs include:

(1) Red LED, emitting at approximately 650 nm with a radiance of $40 \text{ mw/cm}^2/\text{steradian}$;

(2) Green LED, emitting at approximately 540 nm;

and

(3) Blue LED, emitting at approximately 450 nm.

Although fluorescent and radioactive detection methods form the basis of the preferred approaches, other detection procedures are contemplated. Chemiluminescence can be used as the detection method. Interaction of specific (cleaved) blocking groups with immobilized luminol derivatives could also be detected spectroelectrochemically.

In another approach, using mass spectrometric detection, the solution containing cleaved blocking groups or nucleotides is directly injected into a field ionization mass spectrometer. Identification of the

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particular nucleotide incorporated or cleaved is achieved by monitoring the relative abundance of molecular ion peaks corresponding to the specific nucleotides or blocking groups; for example, four distinct acetyl
5 blocking groups differing by one mass unit (replacement of 0 to 3 hydrogens by deuterium) could be detected by monitoring a small "window."

Immobilization of Subject DNA.

10 In the present invention, single stranded subject DNA or its primer is immobilized. One approach to this immobilization is to attach the DNA to a solid substrate. Many of the techniques of modern molecular biology involve immobilization of DNA onto a solid
15 support. DNA and RNA are commonly attached noncovalently through ionic interactions along their length to various types of membranes (Southern, 1975; Maniatis, Fritsch, and Sambrook, 1982; Chuvpilo and Kravchenko, 1984). Similarly, polynucleotides are covalently attached along
20 their length to membranes (Goldberg, et al., 1979), resins (Seed, 1982; Arndt-Jovin, et al., 1975), or plastic (Polsky-Cynkin, et al., 1985). These methods may be employed subject to the caution that this multipoint attachment may, in some cases, introduce interference with
25 the subsequent synthesis of the complementary DNA strand. A single-point covalent attachment of DNA to a solid polymer or glass support is possible. Such single-point methods are preferred for immobilizing the subject DNA, since this leaves the chain free for interactions with the
30 polymerase and similar enzymes used herein.

To effect a single point coupling of DNA to glass or quartz it is often preferred to treat the glass or quartz to assure an inert bond and prevent loss of the DNA during the reactions and rinses carried out in the
35 present method. Pochet et al. (1987) have shown that a very efficient immobilization of DNA occurred on a

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silanized glass surface. Therefore, the inner quartz or glass surface can be advantageously functionalized using silanizing reagents such as triethoxysilylpropylamine or dichlorodimethylsilane. This is followed by covalent
5 attachment of a long-chain alkylamine to these functionalizing groups. The single stranded subject DNA is attached to the long chain amine. The attached single stranded DNA then serves as the template for the formation of the complementary chain.

10 In another embodiment, immobilization is carried out by attaching the subject DNA to a plastic surface. A thin polypropylene chamber wall designed to pass Cerenkov radiation from ^{32}P , for example, can serve as a suitable substrate for DNA immobilization. With a plastic surface,
15 it is preferable to use the method of Kremisky et al. (1987), wherein the surface is coated with streptavidin, to which an alkylbiotinylated oligonucleotide will bind. The immobilized oligonucleotide is annealed to the template DNA as a primer.

20 In addition to retaining the subject single strand DNA by means of immobilizing it to a surface, the subject DNA can also be entrapped by the use of membranes which retain it. In this embodiment, the reaction zone has one or more openings covered with a membrane such as
25 an ultrafiltration membrane, for example, Amicon's PM-5 or PM-10 membranes which have nominal molecular weight cut offs of 5000 and 10,000 respectively. That is, they are capable of passing materials having molecular weights of less than 5,000 and 10,000 respectively while retaining
30 materials above these sizes. Other ultrafiltration or dialysis membranes such as those marketed by Dow or Abcor can also be used. In this embodiment, the single stranded DNA is suspended in liquid in the reaction zone. The labeled and unlabeled dNTPs and other coupling reagents
35 are flowed into the zone. Materials are removed from the zone through such a filter which retains the DNA chains.

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In this method, the polymerase or other enzyme which is used to effect coupling is generally of a size to be retained by the membrane. This scheme works for chemical but not enzymatic deblocking, since in enzymatic
5 deblocking the polymerase and phosphatase must be cycled separately through the cell.

In an alternative embodiment the DNA can be immobilized on particles of resin or polymer microspheres and these particles retained within the chamber. In this
10 embodiment, the filter material is unimportant as long as the DNA is attached to resin particles which are of a size that cannot penetrate the filter pores. There are several methods that couple DNA to resins through the 5' terminus (Pochet, 1987; Polysky-Cynkin, 1985). For example,
15 oligonucleotides or polynucleotides are linked through their 5' end to cellulose (Gilham, 1968; Clerici et al. 1979), Sephacryl (Langdale and Malcolm, 1985), or latex microspheres (Kremsky et al., 1987). In these methods, the DNA is available for interactions with other nucleic
20 acids or proteins. Of particular interest for our application is the method of Goldkorn and Prockop (1986) for covalent coupling of DNA to oligo(dT)-cellulose. Alternatively, the DNA is coupled covalently to streptavidin-agarose beads by an alkylbiotinylated
25 oligonucleotide (Kremsky et al., 1987).

In yet another embodiment, the single-stranded DNA is coupled to DBM paper such as a filter in the presence of a protecting strand. After coupling, the protecting strand is released, leaving the immobilized
30 template and priming site free for successive enzymatic reactions (Hansen et al., 1987). This method and the other single-point methods described above are useful for immobilizing DNA while leaving it free for interactions with enzymes used in DNA sequencing.

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ExamplesExample 1Synthesis of 3'-PO₃[³²P] Thymidine Triphosphate:

5 To a stirred solution of phosphorus trichloride (³²P) (75 mmole) and N-methyl morpholine (750 mmole, Aldrich) in 750 ml dry methylene chloride (CH₂Cl₂) is added 1,2,4-triazole (250 mmole) at room temperature. The reaction mixture is stirred one hour, cooled to 0°C and 15 mmole of 5'-dimethoxytrityl thymidine I (Sigma) in 200 ml of anhydrous acetonitrile is added dropwise over 30 minutes. (See Reaction Scheme 3 given in Figure 7). The solution is stirred an additional 30 minutes, and poured into 600 ml of 1M triethylammonium bicarbonate (TEAB, pH, 15 8.5). The organic layer is separated and the aqueous layer washed with 2 x 200 ml CH₂Cl₂. The combined CH₂Cl₂ extracts are dried over magnesium sulfate (MgSO₄), filtered and evaporated to dryness under vacuum at room temperature. The crude 5'-dimethoxytrityl-3'-thymidine 20 H-phosphonate II is then treated with 2% benzenesulfonic acid in CH₂Cl₂:methanol (MeOH) (7:3) (200 ml) for one hour. The solution is washed with 10% sodium bicarbonate (NaHCO₃) and water, dried over magnesium sulfate and evaporated to dryness. The crude 3'-thymidine- 25 H-phosphonate III is recrystallized from ethanol/ether.

To a solution of 1 ml of phosphorus oxytrichloride (POCl₃) in 30 ml of triethylphosphate at 0°C is added 10 mmole of the 3'-thymidine H-phosphonate. The mixture is stirred for 12 hours at 4°C, neutralized 30 with NaHCO₃ solution, and added to 150 ml water. The aqueous solution is washed with benzene (2 x 100 ml) and ether (2 x 100 ml), and diluted to 0.8 liters with water and charged on a 2.5 x 50 cm column of DEAE-cellulose. The products are eluted using a linear gradient of pH 8.5 35 ammonium bicarbonate solution (0.05 to 0.25 M). The fractions collected are analyzed by HPLC to determine the

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desired product-containing fractions, and these are evaporated to dryness under vacuum. The residue is repeatedly re-evaporated with water to remove salts.

The 5'-monophosphate IV (16 mmole) is then dissolved in 30 ml of dimethylformamide (DMF) and treated with N,N'-carbonyldiimidazole (30 mmole) at room temperature for one hour. The reaction is quenched by addition of 5 ml methanol, and 60 ml of a 0.5M solution of bis(tri-n-butyl-ammonium) pyrophosphate in DMF is added dropwise over 10 minutes. After stirring for 24 hours, the solution is diluted with water to 1 liter and treated with 100 ml of a solution of 0.1 M iodine (I₂) in 5% pyridine/water. After one hour, the solution is deposited on a DEAE-cellulose column from Sigma (5x50cm) or Sephradex from Pharmacia. The column is washed with water and eluted with triethylammonium bicarbonate solution (0.05 to 0.5M). The 5'-triphosphate-3'-phosphate thymidine product V is obtained by evaporation of the appropriate fractions collected.

20

Example 2

Synthesis of 3'-labelled (fluorescent) thymidine triphosphate

A solution of 5-dimethoxytrityl thymidine I (2.5 mmole) in 10 ml dry pyridine is treated with succinic anhydride (8 mmole) at 4°C for 24 hours. Cold water (150ml) is added, and after 30 minutes the solution is filtered. The washed, dried, precipitate is taken up in 30 ml CH₂Cl₂, extracted with water (2 x 25ml), dried over MgSO₄ and evaporated to dryness. (See Reaction Scheme 4 shown in Figure 8.)

The 5'-dimethoxytrityl-thymidine 3'-succinate VI (2mmole) is dissolved in 15 ml dry CH₂Cl₂, cooled to 0°C and treated with a fivefold excess of N,N'-dicyclohexylcarbodiimide and N-hydroxybenzotriazole. After one hour, an equivalent amount of the fluorescent labeling group

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containing a pendant amino function, dansylcadaverine, is added and the solution stirred for 8 hours at 10°C. The solution is then washed with water (2 x 10 ml). The CH₂Cl₂ layer is dried over MgSO₄, and evaporated to dryness to yield the product VII. Removal of the dimethoxytrityl protecting group and conversion to the 5'triphosphate VIII is accomplished in the same manner as described for the 3'-phosphate thymidine triphosphate V.

This reaction is carried out in similar fashion using the other three nucleosides to give the corresponding labeled materials.

Example 3

Quartz Surface Immobilization of Subject DNA

Four 25 microliter volume quartz cuvette reaction chambers are prepared. These chambers are configured like chamber 32 in Figure 3 with the exception that they use their inner walls as the surface to which the DNA is affixed.

The inner surfaces are cleaned and dried. Triethoxysilylpropylamine (5 microliter in 20 microliter CHCl₃) is added and held at 5°C for 120 minutes under anhydrous conditions. This couples the triethoxysilylpropylamine to the surface and gives an amine character to the surface.

The subject DNA is then attached to the amine surface. This is carried out by first attaching a long chain alkyl amine (n-octylamine) to the base at the 5' end of the subject DNA molecule or to the base at the 5' end of a suitable primer, such as an M13 primer for example the 17-mer dGTAAAACGACGGCCAGT, and then joining the alkylamine to the aminopropylsilane surface groups by reaction with glutaraldehyde (1.5 equivalents, 25°C, 120 minutes). Other functional groups pendant to the base moiety or attached to the 5' position can also be used [for example: aldehydes or carboxylic acids (Kremsky et

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al)] for covalent immobilization on derivatized quartz or glass surfaces.

Example 4

5 Incorporation of Labeled Nucleotide Analogs into DNA

The 25 microliter reaction zones are charged with a reaction mixture which contains three Units of SequenaseTM enzyme. The reaction mixture also contains an appropriate buffer for this enzyme (20 mM Tris-HCl pH 7.5,
10 10 mM MgCl₂, 25 mM NaCl, 0.01 M dithiothreitol), the single-stranded primed subject DNA is present at a concentration of approximately 0.1 M attached to the surface of the reaction chamber at its 5' end, (see Example 3), three unlabeled, 3'-blocked deoxynucleotide
15 triphosphate (dNTP) analogs at a concentration of 1.5 micromolar each, and one 3'-blocked, fluorescently labeled dNTP analog of Example 2 at a concentration of 30 micromolar are each present in each of the four reaction zones. In each zone a different one of the four dNTPs is
20 labeled. The reaction proceeds at room temperature for one minute. Then the reaction zones are drained and rinsed with buffers.

In one embodiment the identity of the added dNTP is determined by exciting the fluorophores present in the
25 one cuvette which incorporated its fluorescently-labeled dNTP. Alternatively, the fluorescent group is removed before measurement.

Example 5

30 Chemical Deblocking

The 2,4-dinitrobenzenesulfonyl fluorescent blocking groups are removed with a deblocking reagent which consists of 0.1 M pyridine/pyridinium chloride buffer (pH 7.8) containing thiourea 0.05 M. The
35 deblocking reaction is allowed to proceed for one minute at 40°C. The reaction chamber is then drained and washed

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twice with 100 mM Tris-HCl buffer, pH 6.5. The release of the fluorescent blocking group is measured in the initial eluate from the reaction chamber using a flow-through cell. Depending on the cell in which the fluorescent
5 group is present, the identity of the nucleotide which has been added to the DNA chain is determined. Similarly, if the blocking group were a dansylcadaverine type ester such as in reaction scheme 4, it could be removed by treatment with 50% methanol/50% water pH 10.0 for one minute.

10

Example 6

Enzymatic Deblocking

The blocking group can also be removed enzymatically.

15 For enzymatic deblocking, the deblocker fed into the reaction chamber contains 100 mM Tris-HCl (pH 6.5) 10 mM MgCl₂, 5 mM 2-mercaptoethanol, and one Unit T4 polynucleotide kinase. The reaction proceeds for one minute at a temperature of 37°C. The 3'-phosphatase
20 activity of T4 polynucleotide kinase converts 3'-phosphate termini to 3'-hydroxyl termini which then serve as primers for further synthesis.

While in these examples, the invention has been shown as practiced in a manual manner with each step being
25 carried out sequentially, it can readily be appreciated that this process can be easily automated. A simple clock mechanism or microprocessor driven timer circuit can be used to actuate a plurality of electrically controlled valves in sequence to add the various reagents for adding
30 building blocks, deblocking and the like with the result that the sequence of the target DNA single strand can be obtained with minimum involvement of lab personnel.

While only a few embodiments of the invention have been shown and described herein, it will become
35 apparent to those skilled in the art that various modifications and changes can be made in the present

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invention to methods to determine the sequence of
deoxyribonucleotides in a deoxyribonucleotide chain (DNA)
without the use of a sequencing gel without departing from
the spirit and scope of the present invention.

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What is Claimed:

1. A method for determining the sequence of deoxyribonucleotides in a subject single stranded deoxyribonucleic acid (DNA) molecule comprising:
5 synthesizing, in the presence of the subject DNA molecule, the complementary DNA molecule, the synthesizing being carried out in a stepwise serial manner in which the identity of each deoxynucleotide triphosphate incorporated
10 into the complementary DNA molecule is determined subsequent to its incorporation.
2. The method of claim 1 wherein the synthesizing of the complementary DNA molecule is carried
15 out enzymatically.
3. The method of claim 1 wherein the synthesizing of the complementary DNA molecule is carried out with addition occurring at the 3'-OH position of the
20 complementary DNA molecule.
4. The method of claim 3 wherein each deoxynucleotide triphosphate as incorporated into the complementary DNA molecule is modified to contain a
25 blocking group at its 3'-OH position.
5. The method of claim 4 wherein the blocking group is removed from each deoxynucleotide triphosphate after it has been incorporated into the complementary DNA
30 molecule.
6. The method of claim 1 wherein the identity of each deoxynucleotide triphosphate incorporated into the complementary DNA molecule is determined by identifying at
35 least one reporter group associated with at least one of the four deoxynucleotide triphosphates.

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7. The method of claim 1 wherein the synthesizing of the complementary DNA molecule includes contacting the subject single stranded DNA molecule with all four deoxynucleotide triphosphates under conditions
5 such that the deoxynucleotide triphosphate complementary to the next deoxynucleotide in the subject strand is uniquely incorporated into the complementary DNA molecule.

8. The method of claim 7 wherein the contacting
10 is carried out in a single reaction zone.

9. The method of claim 7 wherein the subject single stranded DNA is contacted with all four deoxynucleotide triphosphates.
15

10. The method of claim 7 wherein the subject single stranded DNA is simultaneously contacted with all four deoxynucleotide triphosphates.

11. The method of claim 10 wherein the contacting is carried out in a single reaction zone.
20

12. The method of claim 10 wherein the contacting is carried out with each of the four
25 deoxynucleotide triphosphates associated with reporter groups distinguishing one from another and wherein the determination of the particular deoxynucleotide triphosphate incorporated is accomplished by identifying the particular reporter group associated therewith.

13. The method of claim 12 wherein the contacting is carried out in a single reaction zone.
30

14. The method of claim 13 wherein the reporter
35 group remains associated with the deoxynucleotide triphosphate after the deoxynucleotide triphosphate is

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incorporated into the complementary DNA molecule such that as each deoxynucleotide triphosphate is incorporated the cumulative reporter signals increase.

5 15. The method of claim 14 wherein the synthesizing of the complementary DNA molecule is carried out enzymatically with addition occurring at the 3'-OH position of the complementary DNA molecule.

10 16. The method of claim 15 wherein the subject single stranded DNA molecule and the growing complementary DNA molecule are immobilized in the reaction zone.

15 17. The method of claim 16 wherein the immobilization of the subject single stranded DNA molecule and the growing complementary DNA molecule is accomplished by enclosing the molecules with porous membranes having pores which are too small for the molecules to pass through.

20 18. The method of claim 16 wherein the immobilization of the subject single stranded DNA molecule and the growing complementary DNA molecule is accomplished by attaching the molecules to a surface within the
25 reaction zone.

19. The method of claim 13 wherein the reporter group is disassociated from the complementary DNA molecule prior to the addition of the next deoxynucleotide
30 triphosphate such that the reporter signal noted when said next deoxynucleotide triphosphate is added is uniquely related to said next deoxynucleotide triphosphate.

20. The method of claim 19 wherein the
35 synthesizing of the complementary DNA molecule is carried

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out enzymatically with addition occurring at the 3'-OH position of the complementary DNA molecule.

21. The method of claim 20 wherein the subject
5 single-stranded DNA molecule and the growing complementary DNA molecule are immobilized in the reaction zone.

22. The method of claim 21 wherein the
immobilization of the subject single stranded DNA molecule
10 and the growing complementary DNA molecule is accomplished by enclosing the molecules with porous membranes having pores which are too small for the molecules to pass through.

23. The method of claim 22 wherein the
immobilization of the subject single stranded DNA molecule
and the growing complementary DNA molecule is accomplished
by attaching the molecules to a surface within the
reaction zone.

24. The method of claim 19 wherein each
deoxynucleotide triphosphate as incorporated into the
complementary DNA molecule is modified to contain a
blocking group at its 3'-OH position and the blocking
25 group is removed from each deoxynucleotide triphosphate after it has been incorporated into the complementary DNA molecule.

25. The method of claim 24 wherein the reporter
30 group is associated with the blocking group.

26. The method of claim 25 wherein the reporter
group is a radiolabel.

27. The method of claim 25 wherein the reporter
35 group is a fluorolabel.

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28. The method of claim 25 wherein the reporter group is identified while associated with the complementary DNA molecule.

5 29. The method of claim 25 wherein the reporter group is identified after being dissociated from the complementary DNA molecule.

10 30. The method of claim 7 wherein the synthesizing is carried out in four parallel reaction zones, each having the four deoxynucleotide triphosphates contained therein and each having a different one of the four deoxynucleotide triphosphates associated with a reporter group.

15 31. The method of claim 30 wherein the reporter groups with which the four deoxynucleotide triphosphates are associated are from one to four different reporter groups.

20 32. The method of claim 30 wherein the reporter groups with which the four deoxynucleotide triphosphates are associated are a single reporter group.

25 33. The method of claim 32 wherein the reporter group remains associated with the deoxynucleotide triphosphate after the deoxynucleotide triphosphate is incorporated into the complementary DNA molecule such that as each deoxynucleotide triphosphate is incorporated the
30 cumulative reporter signals increase.

35 34. The method of claim 33 wherein the synthesizing of the complementary DNA molecule is carried out enzymatically with addition occurring at the 3'-OH position of the complementary DNA molecule.

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35. The method of claim 34 wherein the subject single stranded DNA molecule and the growing complementary DNA molecule are immobilized in the reaction zone.

5 36. The method of claim 35 wherein the immobilization of the subject single stranded DNA molecule and the growing complementary DNA molecule is accomplished by enclosing the molecules with porous membranes having pores which are too small for the molecules to pass
10 through.

37. The method of claim 35 wherein the immobilization of the subject single strand DNA molecule and the growing complementary DNA molecule is accomplished
15 by attaching the molecules to a surface within the reaction zone.

38. The method of claim 32 wherein the reporter group is disassociated from the complementary DNA molecule
20 prior to the addition of the next deoxynucleotide triphosphate such that the reporter signal noted when said next deoxynucleotide triphosphate is added is uniquely related to said next deoxynucleotide triphosphate.

25 39. The method of claim 38 wherein the synthesizing of the complementary DNA molecule is carried out enzymatically with addition occurring at the 3'-OH position of the complementary DNA molecule.

30 40. The method of claim 39 wherein the subject single stranded DNA molecule and the growing complementary DNA molecule are immobilized in the reaction zone.

35 41. The method of claim 40 wherein the immobilization of the subject single stranded DNA molecule and the growing complementary DNA molecule is accomplished

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by enclosing the molecules with porous membranes having pores which are too small for the molecules to pass through.

5 42. The method of claim 41 wherein the immobilization of the subject single stranded DNA molecule and the growing complementary DNA molecule is accomplished by attaching the molecules to a surface within the reaction zone.

10 43. The method of claim 38 wherein each deoxynucleotide triphosphate as incorporated into the complementary DNA molecule is modified to contain a blocking group at its 3'-OH position and the blocking
15 group is removed from each deoxynucleotide triphosphate after it has been incorporated into the complementary DNA molecule.

20 44. The method of claim 43 wherein the reporter group is associated with the blocking group.

 45. The method of claim 44 wherein the reporter group is a radiolabel.

25 46. The method of claim 44 wherein the reporter group is a fluorolabel.

 47. The method of claim 44 wherein the reporter group is identified while associated with the
30 complementary DNA molecule.

 48. The method of claim 44 wherein the reporter group is identified after being dissociated from the complementary DNA molecule.

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49. A method for determining the sequence of deoxyribonucleotides in a subject single stranded deoxyribonucleotide (DNA) molecule comprising:

- 5 (a) synthesizing, in the presence of the subject DNA molecule, the complementary DNA molecule, the synthesizing being carried out in a stepwise serial manner in which the identity of each deoxynucleotide triphosphate incorporated into the complementary DNA molecule is determined subsequent to its incorporation;
- 10 (b) translating the identity of each deoxynucleotide triphosphate incorporated into the complementary molecule to the identity of its corresponding complement present in the subject molecule; and
- 15 (c) tabulating the identities of the corresponding complements thereby giving rise to the deoxyribonucleotide sequence of the subject DNA.

50. A method for determining the sequence of deoxyribonucleotides in a subject single stranded deoxyribonucleotide (DNA) molecule comprising:

- 20 (a) synthesizing, in the presence of the subject DNA molecule an initial region of the complementary DNA molecule, the synthesizing being carried out in a stepwise serial manner in which the identity of each deoxyribonucleotide triphosphate incorporated into the complementary DNA molecule is determined subsequent to its incorporation;
- 25 (b) tabulating the identities of the deoxyribonucleotides incorporated into the initial region of the complementary DNA molecule;
- 30 (c) removing the initial region of the complementary DNA molecule for the subject single stranded DNA molecule;

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(d) separately synthesizing a DNA primer molecule corresponding in sequence to at least a part of the initial region of the complementary DNA molecule;

(e) annealing the DNA primer molecule to the
5 subject single stranded DNA molecule;

(f) synthesizing, from the DNA primer molecule the next region of the complementary DNA molecule;

(g) tabulating the identities of the
deoxyribonucleotides incorporated into the next region of
10 the complementary DNA molecule; and

(h) repeating steps c, d, e, f and g as needed to determine the entire structure of the subject single stranded DNA molecule.

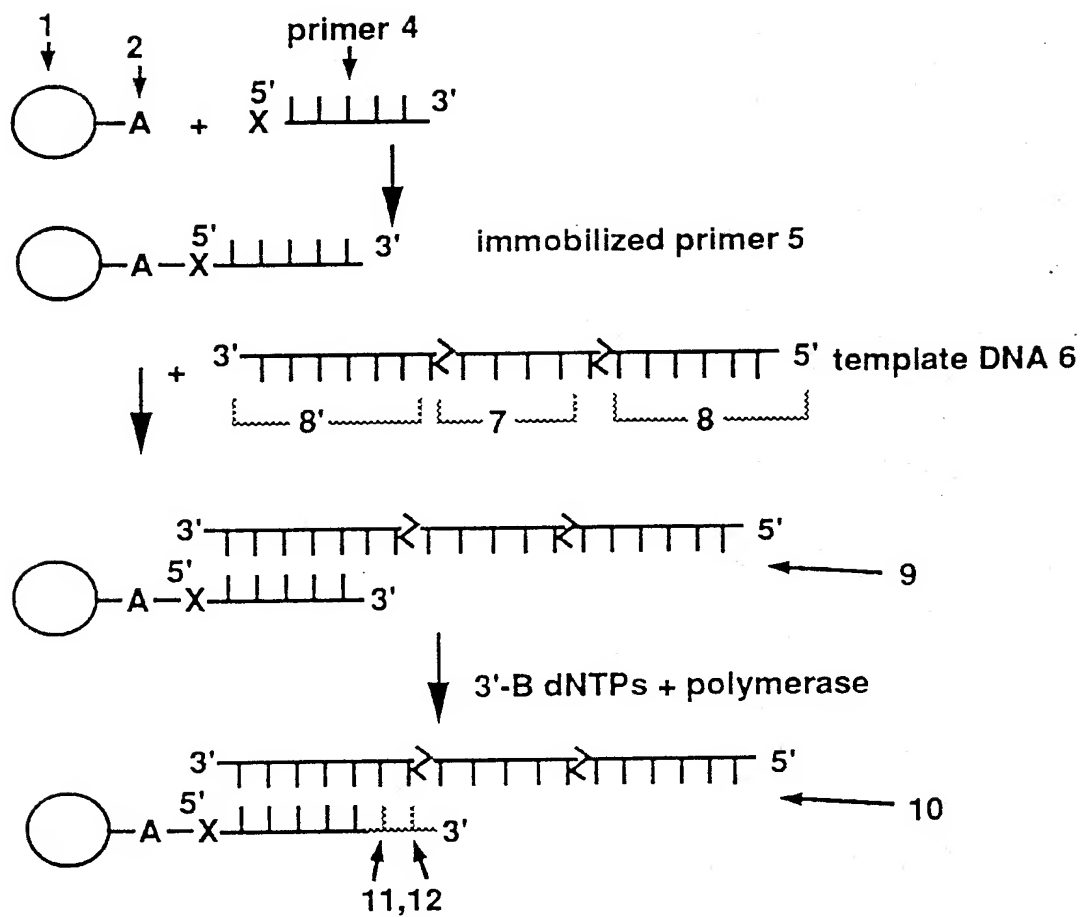
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35

**FIG. 1A**

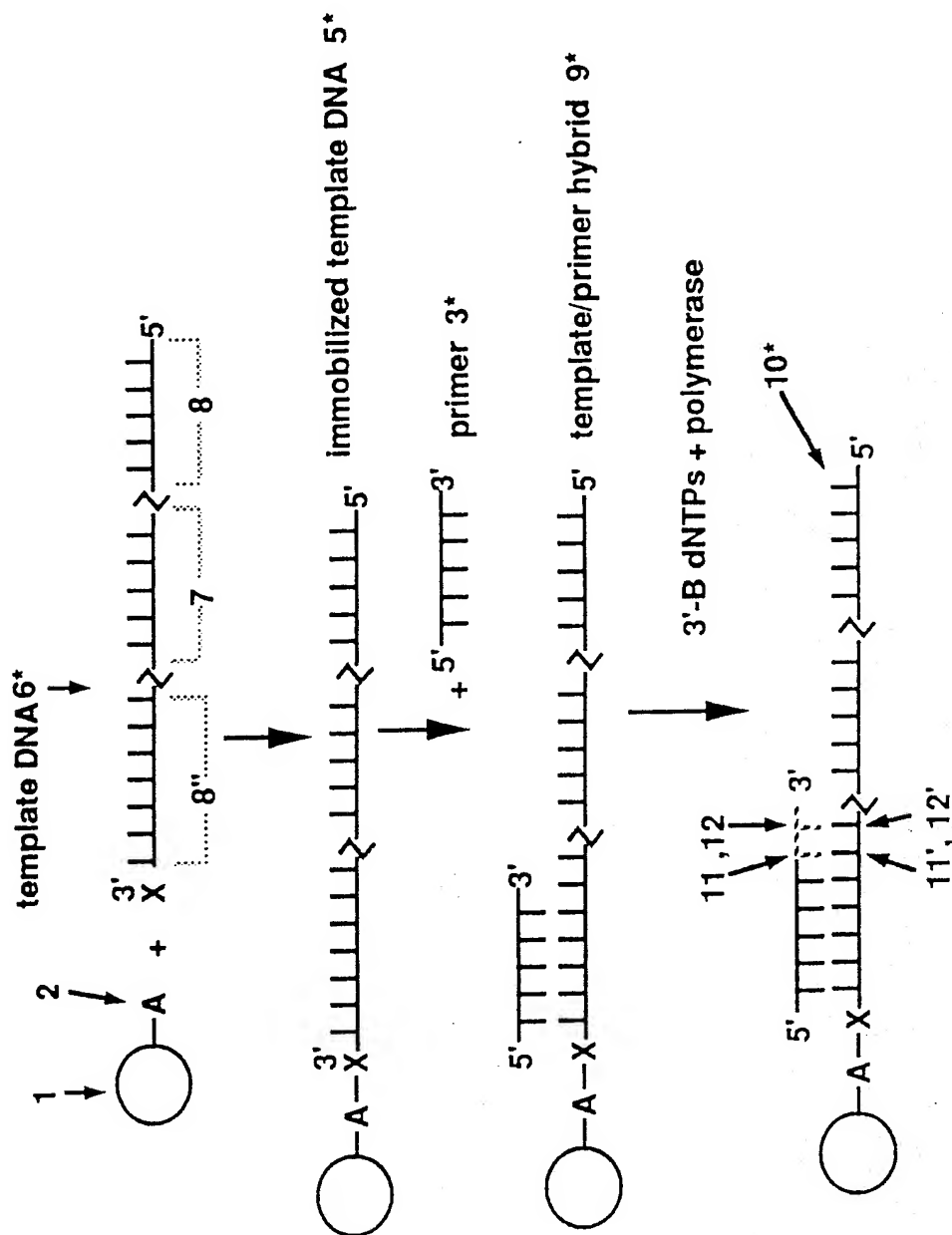


FIG. 1B

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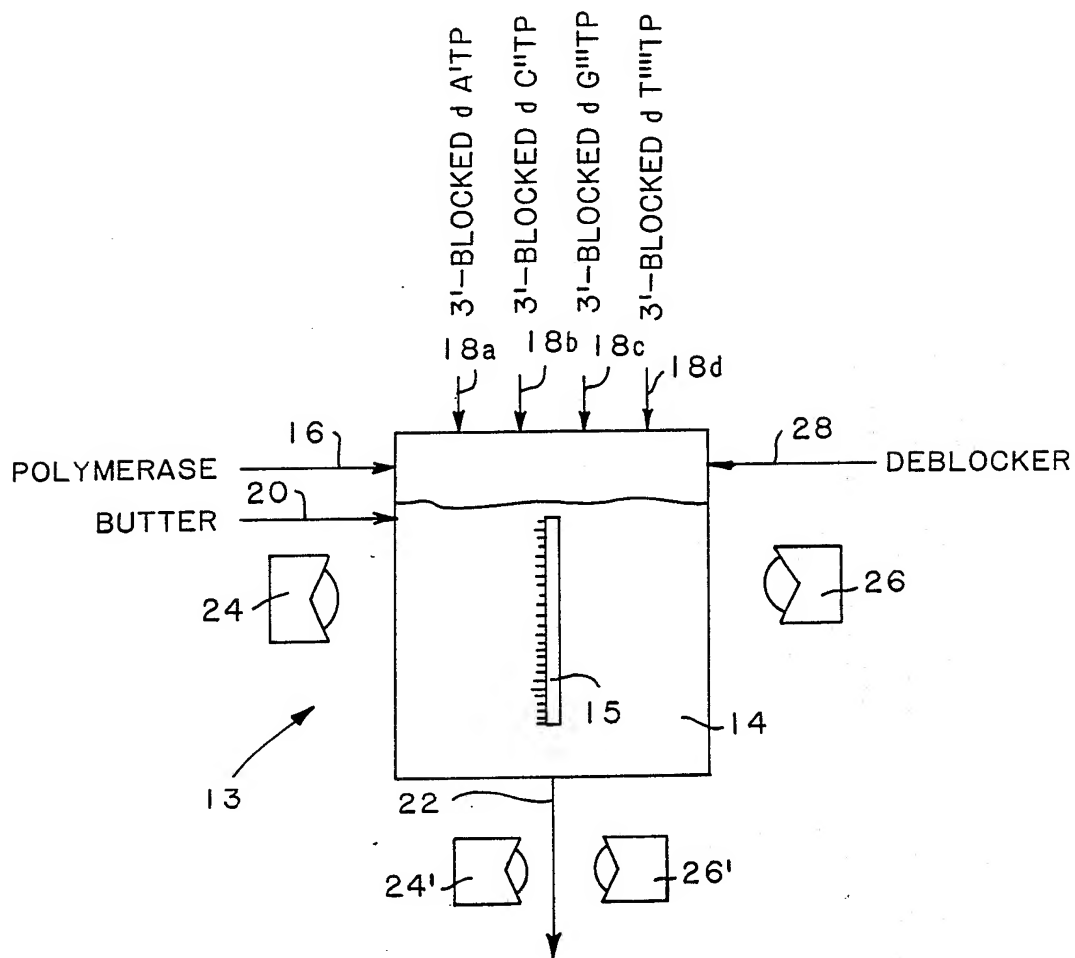


FIG. 2

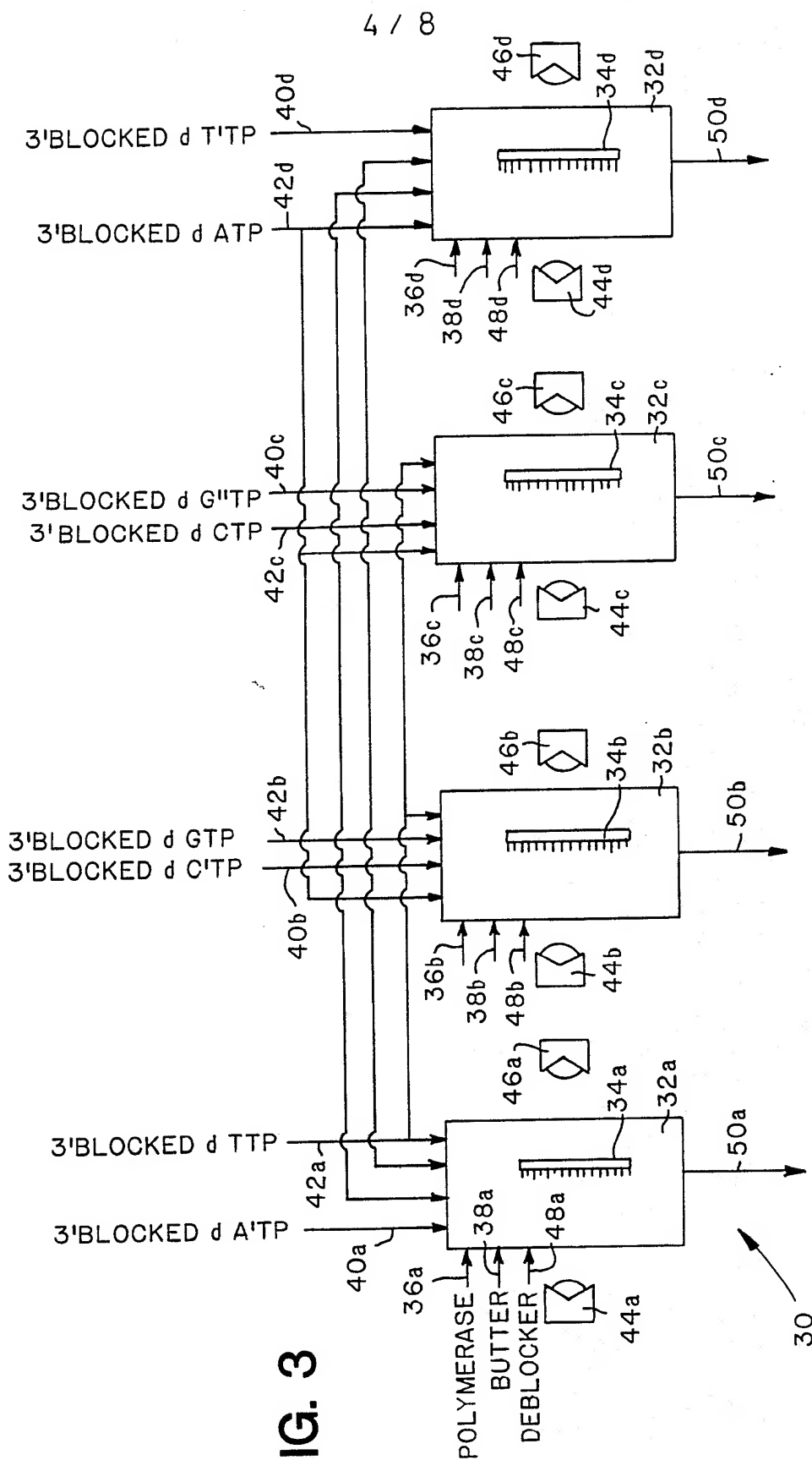


FIG. 3

FIG. 4

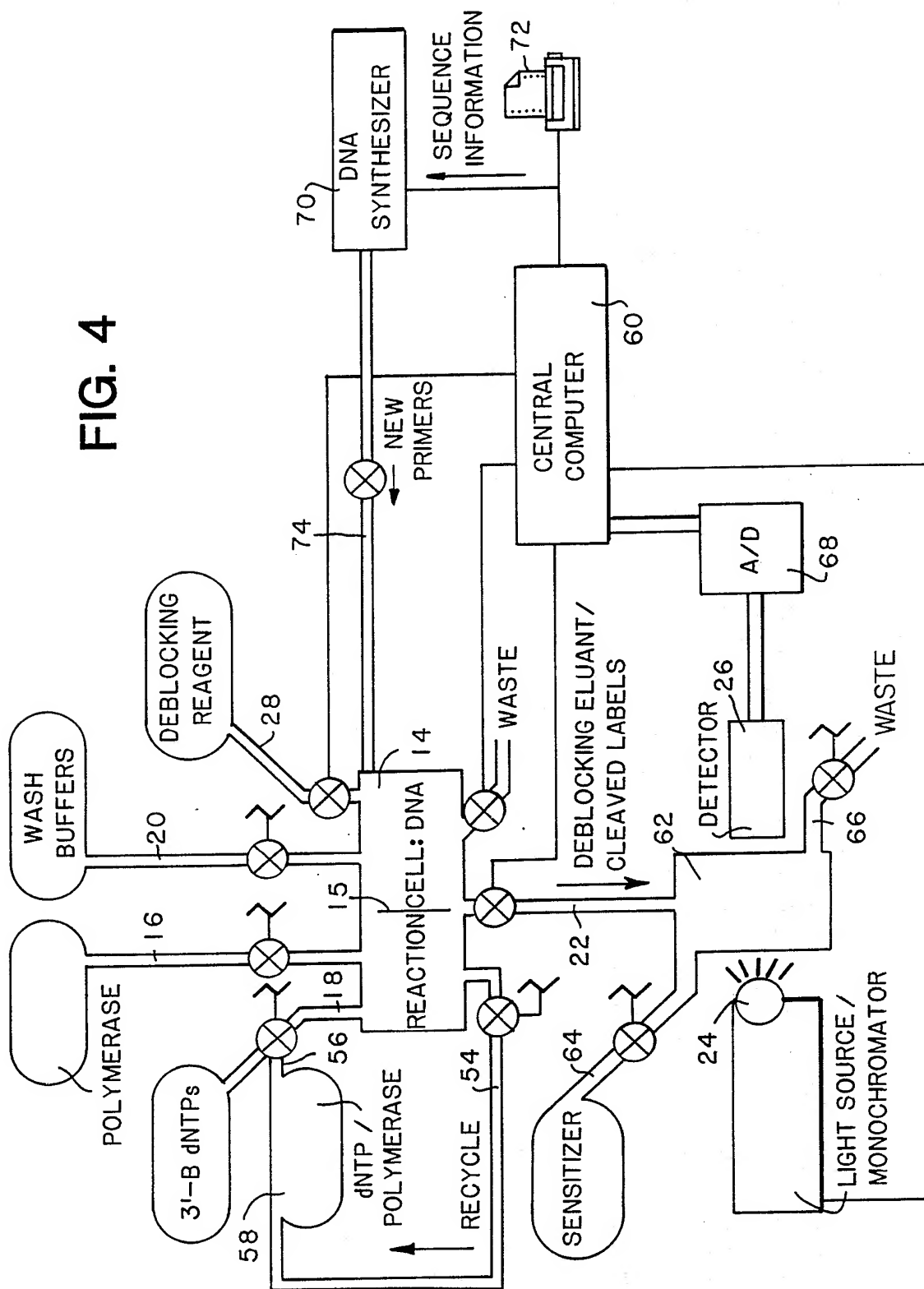
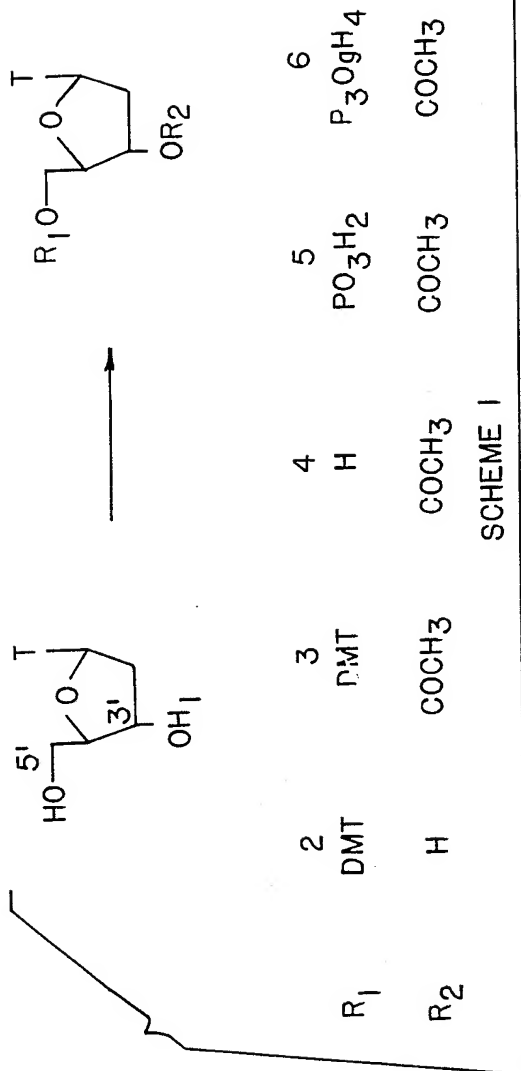


FIG. 5



R ₁	2	7	8	9	10	11
	DMT	DMT	H	PO ₃ H ₂	P ₃ OgH ₄	P ₃ OgH ₄
R ₂	H	PO ₂ H	PO ₂ H	PO ₂ H	PO ₂ H	PO ₃ H ₂

FIG. 6

SCHEME II

FIG. 7

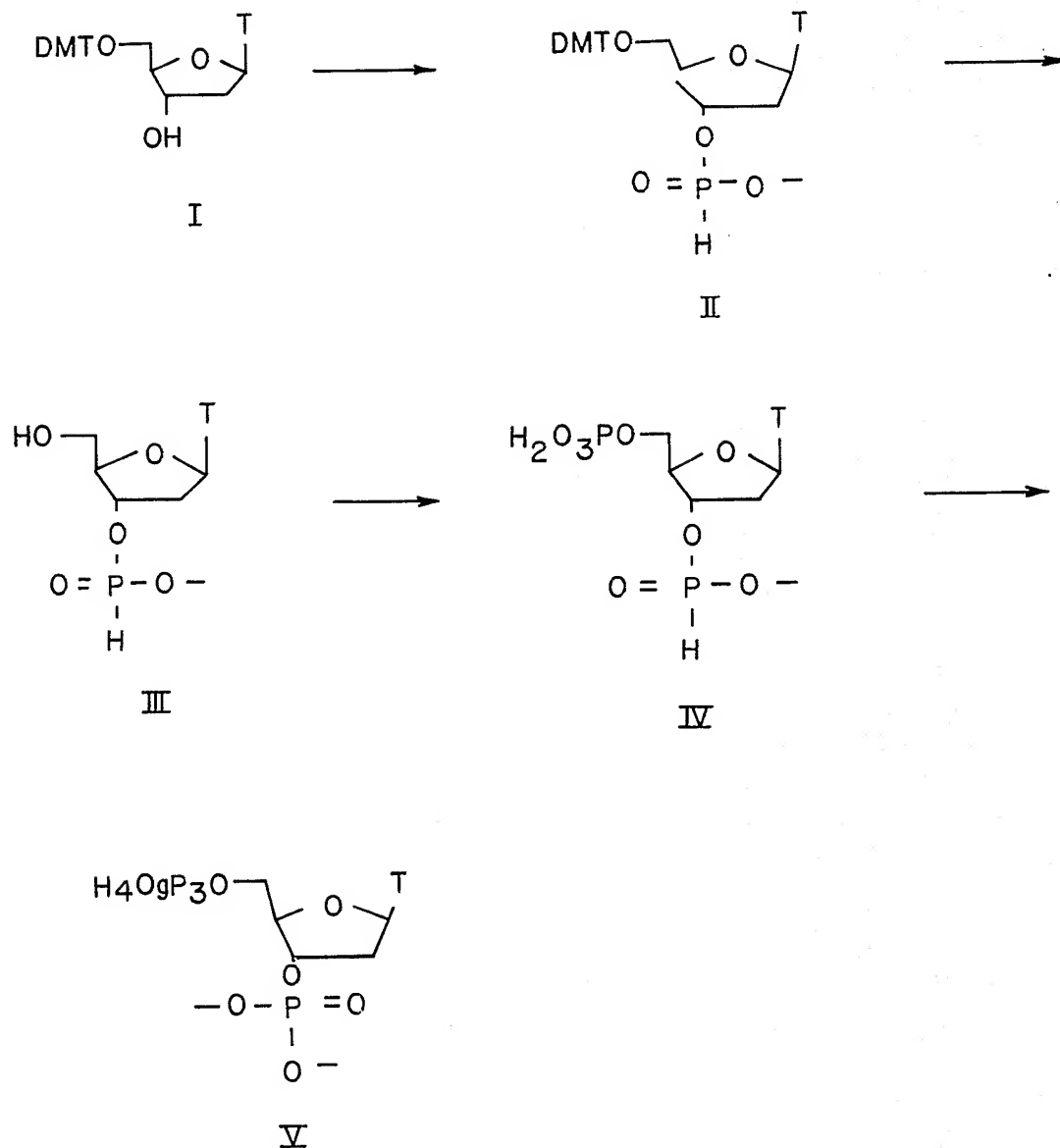
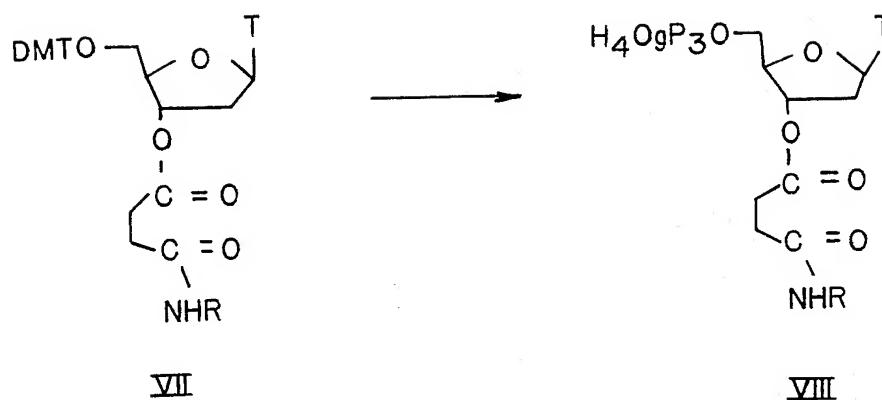
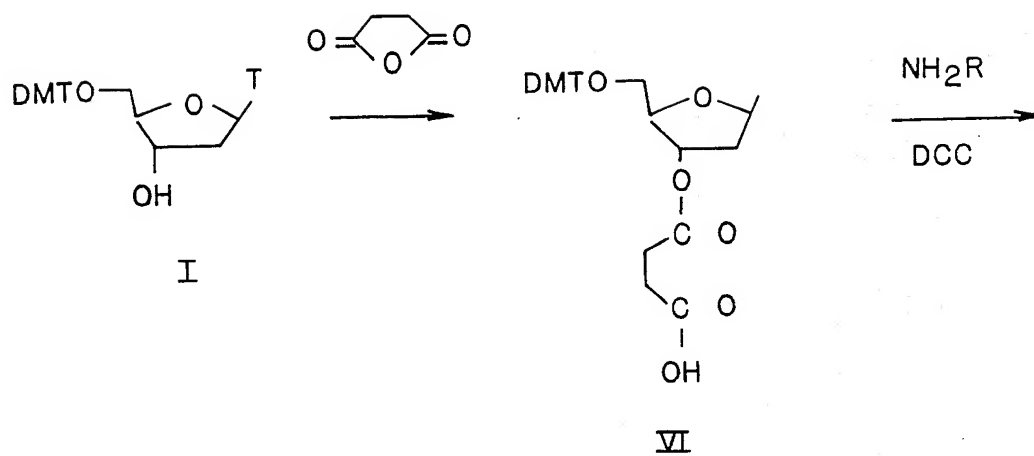
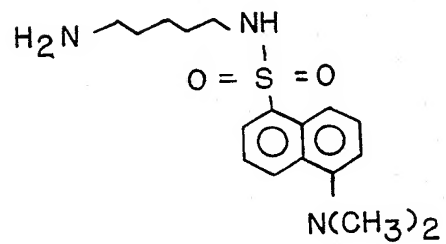


FIG. 8




e.g. $\text{NH}_2\text{R} =$



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/06178

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ C 12 Q 1/68, C 12 P 19/34, // C 07 H 21/00, C 07 H 21/04, IPC ⁵ : G 01 N 35/08		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 12 Q, C 07 H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁶	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO, A, 89/09283 (E. HYMAN) 5 October 1989 see abstract; claims	1-3, 7-9, 49,
Y	--	30-32
X	EP, A, 0223618 (NEW YORK MEDICAL COLLEGE) 27 May 1987 see the whole document	1-3, 6-9, 49
Y	--	4, 5, 10-13, 30-32
Y	EP, A, 0219342 (GENENTECH, INC.) 22 April 1987 see claim 1	4, 5
	--	
./.		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28th February 1991	04 APR 1991	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 MISS T. TAZELAAR	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹ with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	BioTechniques, vol. 5, no. 4, 1987, (Watick, Mass., US); C. Connell et al.: "Automated DNA sequence analysis", pages 342-348, see abstract and Introduction (cited in the application) --	10-13
P,X	WPIL, File Supplier, AN = 90-105130 (14), Derwent Publications Ltd, (London, GB), & JP, A, 2057978 (TAKARA SHUZO K.K.) 27 February 1990 see abstract --	1
P,X	WPIL, File Supplier, AN = 90-250698 (33), Derwent Publications Ltd, (London, GB), & JP, A, 2174700 (TAKARA SHUZO K.K.) 6 July 1990 see abstract --	1
E	WO, A, 90/13666 (AMERSHAM INTERNATIONAL PLC) 15 November 1990 see the whole document --	1-3,7-9,49
A	Nucleic Acids research, vol. 15, no. 7, 1987, J.N. Kremsky et al.: "Immobilization of DNA via oligonucleotides con- taining at aldehyde or carboxylic acid group at the 5' terminus", pages 2891-2909, see the whole article, especially the abstract (cited in the application) --	16,18,21,23, 35,37,40,42
A	WO, A, 89/03432 (UNITED STATES DEPART- MENT OF ENERGY) 20 April 1989 -----	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9006178
SA 42222

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 25/03/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8909283	05-10-89	US-A- 4971903 AU-A- 3354889	20-11-90 16-10-89
EP-A- 0223618	27-05-87	US-A- 4863849 JP-A- 62085863	05-09-89 20-04-87
EP-A- 0219342	22-04-87	US-A- 4959463 JP-A- 62116593	25-09-90 28-05-87
WO-A- 9013666	15-11-90	None	
WO-A- 8903432	20-04-89	US-A- 4962037 EP-A- 0381693	09-10-90 16-08-90